Identification of the source of antibiotic-resistant *Escherichia coli* in an urban river basin

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**Abstract**

To identify the source of antibiotic-resistant *Escherichia coli* (*E. coli*), a cluster analysis for multiple antibiotic resistance (defined as resistance to more than two kinds of antibiotics) patterns was performed in the Tama River basin, Tokyo, Japan. In this study, the multiple antibiotic resistance patterns in tributary and downstream Tama River were the most similar to those in raw sewage. On the other hand, upstream sites showed different pattern from raw sewage. These results show that the antibiotic-resistant *E. coli* was dominantly attributable to the anthropogenic source of urbanized watershed areas, which usually transport it through the sewer systems such as combined sewer overflows and stormwaters. The cluster analysis for multiple antibiotic resistance patterns used in this study might be a simple and useful approach to identify the source of antibiotic-resistant *E. coli* among different locations.

**Keywords:** antibiotic-resistant *Escherichia coli*; multiple antibiotic resistance pattern; raw sewage; urban river basin

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**1. Introduction**

Worldwide, concern for fecal contamination of urban water bodies and water courses is increasing. However, the complex pathways of contamination make it difficult to identify the origins and quantitative contributions of various sources of fecal contaminants. In urban environments, bacteria and viruses originating from human and animal feces are generally transported through the sewer systems to centralized wastewater treatment facilities before being partially treated and discharged into receiving water bodies (Sturtevant 1969; Sturtevant et al. 1971; Iwane et al. 2001). Moreover, it has been reported that ecological effects of fecal contamination might become even more substantial due to antibiotic resistance of fecal bacteria (e.g., *E. coli*) because their capability of transferring the antibiotic resistance genes to the neighboring bacterial colonies (Tauxe et al. 1989; Mezrooui and Baleux 1994; Arvanitidou et al. 1997; Levy 1997; Reinthaler et al. 2003; Salyers et al. 2004).

Recently, there has been a growing awareness that the individual benefits of antibiotic use are diminished by a societal cost, which includes the threat of antibiotic resistance among bacterial pathogens. Although initially more prevalent in hospital settings, the prevalence of drug resistance in biologic community settings is also rising among bacterial pathogens, particularly *E. coli* (Gupta et al. 1999a & 1999b). Accordingly, waters that receive waste treatment and raw sewage discharge

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are susceptible to the effects of contamination by antibiotic-resistant bacteria throughout the sewer systems associated with watershed communities. To date, identification of the individual sources of contaminants has been used to estimate the effect of fecal contamination on rivers. However, it is difficult to investigate the individual sources of fecal contaminants and their contributions to rivers due to the complexity of their transport mechanisms.

In this study, we try to identify predominantly natural or anthropogenic sources using multiple antibiotic resistance patterns in raw sewage and river waters in the Tama River basin in Tokyo Metropolitan area in Japan.

2. Materials and Methods

2.1 Study area

Eleven stations of the Tama River basin (Station 1: 35°78'15.29"N, 139°23'13.78"E to Station 12: 35°58'53.96"N, 139°66'87.90"E) were selected for the study area, which is located in Tokyo Metropolitan areas and in Kawasaki City areas (Figure. 1). Approximately 138 km long, the Tama River has its source in Mt. Kasatori (elevation: 1953 m, in Yamanashi and Saitama Prefectures) and flows toward the southeast into Tokyo Bay. The annual average air temperature in Tokyo was 16.0 ± 0.7°C (average ± SD), ranging from 5.9°C (monthly minimum) in January to 27.1°C (monthly maximum) in August during 1965–2009. Average annual rainfall in the same period was 1485 ± 255 mm, ranging from 49 mm (monthly minimum) in December to 197 mm (monthly maximum) in September (Japan Meteorological Agency 2010).

Ten river water sampling stations were selected throughout the Tama River basin, which were divided by upstream (S1–S3), midstream (S3–S8) and downstream (S8–S10) areas (Figure. 1). In addition, one raw sewage sampling station was selected where was located between S3 and S7 in the midstream Tama River.

Figure 1 The location of sampling stations in the Tama River basin in Tokyo Metropolitan area. Upstream stations: S1 and S2, Midstream stations: S3, S7 and S8, Downstream stations: S9 and S10, tributary sites: S4, S5 and S6. Arrow: raw sewage station
2.2 Experiment

During the daytime within the same day, water samples were collected at the stations in sterilized 100 mL glass bottles once a month between July 2003 and November 2004. All the water sample bottles were immediately placed into a hand-held ice box to keep the temperature below 4°C. The water samples were experimented in the Field Work Facility (a Faculty laboratory) in Faculty of Environmental and Information Studies, Tokyo City University.

Materials for the experiment sterilized by an autoclave (KT-30SD, ALP Co., Ltd., Japan) at 121°C for 20 minutes was as follows; deionized distilled water (AQUARIUS GS-2000, ADVANTEC), micropipette chips, plate count agar (Eiken Chemical Co., Ltd., Japan) and glucose-phosphate media (Eiken Chemical Co., Ltd., Japan) (pH 6.9). Presterilized 90 × 15 mm plastic Petri dishes (PD-915, ADVANTEC) were also used for this experiment. All the collected water samples were diluted 1-100 times with the presterilized water. The experiment was conducted in a clean bench (CVT-840, SIBATA, Japan).

Each 0.1 mL water sample was spread and incubated on a desoxycholate agar plate (Eiken Chemical Co., Ltd., Japan) for 18-20 hr at 44.5 ± 0.2°C (Incubator DRS-620DA, ADVANTEC) (Japan Sewage Works Association 1997). Red-colored colonies appeared on the plates were counted as fecal coliform bacteria. Several fecal bacterial colonies were then taken from the plates with a platinum loop. Each colony was spread and incubated on plate count agar (Eiken Chemical Co., Ltd., Japan) plates for 18 hr at 36±1°C (Incubator DRS-620DA, ADVANTEC). In addition, single colony isolation was performed to produce a pure culture. The isolated fecal coliform colony was diluted by 4.5 mL presterilized water in each test tube. The suspension (0.5 mL) was then diluted with presterilized water (4.5 mL). Thereafter, the suspension (0.5 mL) was transferred into 0.5 mL of LMX Broth (LMX Broth-10, Kanto Chemical Co., Inc., 24283-96) in a test tube, which was then incubated at 35°C for 24 hr (Incubator DRS-620DA, ADVANTEC). The blue colonies fluoresced a bright blue color under long-wave UV light (λ, 366 nm), which identified them as *E. coli* (Dahlén and Linde 1973; Feng and Hartman 1982). The colonies were further characterized as *E. coli* by subculture on plate count agar (Eiken Chemical Co., Ltd., Japan).

Each *E. coli* colony was transferred with a platinum loop into a tube filled with 4 mL glucose-phosphate media (pH 6.9). The test tube was incubated for 3 hr at 35±0.2°C (Incubator DRS-620DA, ADVANTEC) until they contained *E. coli* up to 104 cfu/mL. Each 0.1 mL culture was then streaked onto plate count agar plates (Eiken Chemical Co., Ltd., Japan). Antibiotic resistance testing by the disk diffusion method (Sayah et al. 2005) was conducted for ampicillin (ABPC), amoxicillin-clavulanic acid (ACV), cefixime (CFI), kanamycin (KM), streptomycin (SM), tetracycline (TC), minocycline (MNO), chloramphenicol (CP), nalidixic acid (NA), ofloxacin (OFX), ciprofloxacin (CIP) and trimethoprim-sulfamethoxazole (TMP-SMX), which were commercially supplied Kirby-Bauer (KB) disks (Eiken Co. Ltd., Tokyo, Japan) except SM (Showa disc; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). The disk was then put on the plate within 20 minutes, which was incubated for 16-18 hr at 35°C (IS-2000, ADVANTEC).

2.3 Statistical analyses

A hierarchical cluster analysis was performed using the Ward’s method for grouping the samples with the fecal sources which was based on the similarity of multiple antibiotic resistance patterns, which is an application of widely used approaches of source identification for fecal contaminants (Wiggins 1996; Parveen et al. 1997; Hagedorn et al. 1999).
3. Results

In our antibiotic susceptibility testing, 375 *E. coli* strains in the Tama River and raw sewage samples indicate the resistance against 12 antibiotics, ranging from 0% to 67% (Figure 2). The Tama River can be characterized by high resistance rates to trimethoprim-sulfamethoxazole (24%), ampicillin (16%) and tetracycline (9%). The analysis of antibiotic resistance pattern for *E. coli* in raw sewage shows similar resistance rates for ampicillin (22%), trimethoprim-sulfamethoxazole (21%) and tetracycline (11%). From these results, it seems likely that the fecal contamination in the Tama River is associated with the raw sewage.

In a cluster analysis for multiple antibiotic resistance patterns, 8 stations in the Tama River can be grouped with the raw sewage except station 1 and 2 (Figure 3). The upstream Tama River grouped into Group 1 and Group 2 shows significantly different antibiotic resistance rates (ABPC: \( p < 0.01 \), ACV: \( p < 0.001 \), CFI: \( p < 0.001 \), CIP: \( p < 0.01 \), TMP-SMX: \( p < 0.001 \), one-way ANOVA) compared to other stations (Group 3), including the raw sewage site.

The average rate of antibiotic-resistant *E. coli* in the upstream, midstream, downstream and tributary sites varied from 0% to 54% (Figure 4). In this study, multiple antibiotic resistance patterns in the downstream and tributary Tama River can be clustered with the raw sewage (Figure 5). The upstream and midstream Tama River, grouped into Group 1 and Group 2, indicate significantly different antibiotic resistance rates (ACV: \( p < 0.01 \), CFI: \( p < 0.05 \), TMP-SMX: \( p < 0.01 \), one-way ANOVA) compared to the raw sewage, downstream and tributary sites. Consequently, these results offer evidence that the source of fecal contamination in the downstream and tributary Tama River is closely related to raw sewage inputs in the watersheds.

Figure 2 Multiple antibiotic resistance patterns for *E. coli* in 1 raw sewage and 10 river stations in the Tama River basin during July 2003 – November 2004
Figure 3 The hierarchical cluster analysis for multiple antibiotic resistance patterns (Source data: Figure 2, The Ward’s method of SPSS 19)

Figure 4 Multiple antibiotic resistance patterns for *E. coli* in 1 raw sewage, upstream, midstream, downstream Tama River during July 2003 – November 2004

Figure 5 The hierarchical cluster analysis for multiple antibiotic resistance patterns (Source data: Figure 4, The Ward’s method of SPSS 19)
4. Discussion

This study has tried to identify the source of antibiotic resistant *E. coli* using a cluster analysis for antibiotic resistance patterns (here among raw sewage and river water stations) as a simple approach. In this study, the results of cluster analyses showed a similarity among the multiple antibiotic resistance patterns among the raw sewage and tributary and downstream Tama River sites. This result is also supporting the attribution of the incidence of antibiotic-resistant *E. coli* in the Tama River to discharges into the river via the sewer system of watershed area. The possible routes of the antibiotic-resistant *E. coli* contamination in the sewer system are mainly 1) treated sewer effluents, 2) combined sewer overflows and 3) stormwaters. In addition, there are several relevant data; 1) over 50% of the total flow volume in the downstream Tama River originated from treated sewer effluent (Tokyo Metropolitan Government 2005), 2) respective 24% and 82% of the sewer systems in the western (upstream and midstream areas) and eastern parts (midstream and downstream areas) of the Tama River Basin (Tokyo Metropolitan Government 2005; Ministry of Land, Infrastructure and Transport, Government of Japan 2007) are comprised of the combined sewer systems that can discharge combined sewer overflows with a large amount of fecal bacterial contamination, including antibiotic-resistant *E. coli*, into the river during wet weather, and 3) there is a possibility that the incidence of antibiotic-resistant bacteria in the Tama River originated from the treated wastewater discharge (Iwane et al. 2001).

Although the trimethoprim-sulfamethoxazole was originally developed as a synthetic antibiotic to suppress antibiotic-resistant bacteria (Hitchings 1973; Nelson et al. 1976), in the case of the United States, it has been shown that the therapeutic potency of trimethoprim-sulfamethoxazole declines with the increase in resistance rates (Smilack 1999). Similarly, this study is reporting high resistance rates to trimethoprim-sulfamethoxazole in the Tama River basin. Consequently, now we need more efforts to identify and mitigate the diffusion of antibiotic resistant bacteria.

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References


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